Biosurfactant Production with Glucose as a Carbon Source

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Original scientific paper
Received: September 21, 2004
Accepted: December 10, 2005

A rhamnolipid producing bacterium, *P.aeruginosa MM1011*, was previously over years isolated from crude oil. Isolated strain was identified by morphological, biochemical, physiological, and 16srRNA.[1] The identified *Pseudomonas aeruginosa* was confirmed by Persian Type Culture Collection (PTCC idendification confirmation report No. 1011). Glycolipid production by isolated bacterium using sugar beet molasses as a carbon and energy source, was investigated. *MM1011* was used for the development of a continuous process for biosurfactant production. The active compounds were identified as rhamnolipids. A final medium for production was designed in continuous culture by means of medium shifts, since the formation of surface-active compounds was decisively influenced by the composition and concentration of the medium components. In the presence of yeast extract, biosurfactant production was poor. For the nitrogen-source nitrate, which was superior to ammonium, an optimum carbon-to-nitrogen ratio of ca. 18 existed. The iron concentration needed to be minimized to 27.5 μg of $FeSO_4 \cdot 7H_20$ per g of glucose. A carbon-to-phosphate mass ratio, $\zeta_{c/p} = 20$, obtained the maximum production of rhamnolipids. The final productivity dilution rate diagram indicated that biosurfactant production was correlated to low growth rates (dilution rate below 0.18 h^{-1}). With a medium containing 24.2 g l^{-1} of glucose, a biosurfactant mass concentration (expressed as rhamnolipids) of up to 1.1 g l^{-1} was obtained in the cell-free culture liquid. The rhamnolipid mass concentration was 7.5 mg ml^{-1} and surface tension was reduced to 20 mN m^{-1}.[2,3]

Key words:
Biosurfactant, wild type, *Pseudomonas aeruginosa*, sugar beet Molasses

Introduction

Surfactants and emulsifiers are widely used in the petroleum, pharmaceutical, cosmetic, and food industries. Most of these compounds are chemically synthesized and it is only in the past few decades that surface-active molecules of biological origin have been described. At present, biosurfactants are readily bio- degradable and can be produced from renewable and cheaper substrates, they might be able to replace their chemically synthesized counter parts.[1,2]

Among the heterogeneous group of biosurfactants, the rhamnose-containing glycolipids are produced by *Pseudomonas*. There is a recent increase of interest in the production of biosurfactants beacause of their biodegradability, reduced toxicity compared to synthetic surfactants, and their application in enhanced oil recovery and food emulsification.

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Almost all surfactant currently in use are chemically derived from petroleum. However, interest in microbial surfactant has been steadily increasing in recent years due to their diversity, environmentally friendly characteristics, the possibility of their production through fermentation, and their potential application in such areas as the environmental protection, surplus crude oil recovery, health care, and the food processing industries.[3,4,5] Therefore, it is necessary to know more about the producing microorganism's physiology and the process engineering to develop the technology for these production of molecules, the use of cheap substrates being of utmost importance.[6,7] Interest in microbial surfactants has increased considerably in recent years, especially due to their potential application in enhanced oil recovery. The production of surface-active compounds by microorganisms is well established[1,5,13,19] and has been a matter of discussion at different international meetings.[6,18] Their potential for enhanced oil recovery is based on their application as agents for rock wetting, micellar flooding, emulsification, deemulsification, and viscosity reduction of heavy crude oils.[8] The industrial demand for sur-

factants has grown to about 300 % within the U.S. chemical industry during the last decade. Rapid advances in biotechnology over the past decades have led to considerable interest in the development of biological methods for manufacturing surfactants on the industrial scale. Various types of biosurfactant are synthesized by a number of microbes particularly during their growth on water-immiscible substrates. A majority of biosurfactants are produced by bacteria. Among the bacteria, *Pseudomonas* species is well known for its capability to produce rhamnolipid biosurfactant with potential surface-active properties when grow on different carbon substrates. Carbohydrates were rarely used as carbon and energy source for biosutfactant production with the exceptions of *Arthrobacter sp*.,[21] *Bacillus subtilis*,[3] *Torulopsis bombicola*,[4] and *Pseudomonas aeruginosa*.[9] Although less qualified for spontaneous formation, the production of biosurfactants from carbohydrate substrates offers some advantages as compared with hydrocarbons. From an engineering point of view, hydrocarbon substrates require more sophisticated equipment and more power input to achieve an adequate dispersion of the insoluble hydrocarbons. In addition, the availability of hydrocarbons is limited if applications of biosurfactants other than in enhanced oil recovery are envisaged. *P. aeruginosa* production of surface-active compounds has been reported already.[11,13,15] In these studies, the minimal surface tension of the cell-free culture broth was about 40 mN m^{-1}. The surface activity was always related to a glycolipid moiety, i.e., a rhamnolipid. Two kinds of rhamnolipids were formed by *P. aeruginosa*. One, called R-1,[7] consisted of two molecules of rhamnose and two molecules of P-hydroxydecanoic acid. Later, a second kind of rhamnolipid, R-2, was identified. It was similar to R-1, but the molecule contained only one rhamnose unit.[13] It was demonstrated that both R-1 and R-2 are essential for *P. aeruginosa* growth on hydrocarbons.[14] The rhamnolipids were produced when hydrocarbons, glycerol, glucose, or peptone were the substrate.[11,13–15] Best production was obtained with hydrocarbons or glycerol.[13] For the bulk production of biosurfactants, a primary prerequisite is the definition of culture conditions in which high yields of surface-active compounds are obtained by a particular microorganism. This forms the basis for a realistic estimation of the feasibility of biosurfactant production. We report here on such basic studies which aimed at the design of a continuous process for biosurfactant production by a *P. aeruginosa* strain with glucose as the carbon source.

The objective of this study was to investigate high production of rhamnolipid by growing the newly isolated strain of *Pseudomonas aeruginosa* MM1011 on sugar beet molasses. The emulsification capacity, Rhamnolipid1 (RL1), Rhamnolipid2 (RL2), were studied. The sugar beet molasses consumption with this isolate is 2 % as compare to 7 % in general.

Materials and methods

The bacteria were previously isolated from crude oil. On the basis of prompt emulsifying activity, the best species selected, and Upon 16 srRNA the isolate identified as *Pseudomonas aeruginosa*.[1] The isolated strain was later confirmed by PTCC (PTCC Identification report No. 1011) and therefore is designated as *Pseudomonas aeruginosa MM1011*. The bacteria was maintained at 4 °C on nutrient agar slants and subcultured every two weeks.

The compositions of the media used in this study are listed in Table 1. Cultivation conditions. Growth of microorganisms was developing in a 5 l compact loop (color) bioreactor (developed at this department) equipped with a mechanical foam separator. The temperature was 37 °C, and the pH was controlled at 6.5 with 1 mol l^{-1} KOH. Stirrer and foam separator speeds were set at n = 1,500 and 2,000 min^{-1}, respectively. The working volume was 1.5 l, and the aeration was 2.25 l min^{-1}. The medium optimization was performed at a dilution rate of D_{mass} = 0.10 h^{-1}. Steady states were considered

Table 1 – *Media used for the cultivation of P. aeruginosa in the course of medium optimization*

$m_{glucose} + m_{compounds}$	Medium			
Glucose (g)	4M	3M	2M	1M
$(NH_4)_2SO_4$ (mg)	137.5	137.5	220	220
$NaNO_3$ (mg)	-----	-----	162	110
K_2HPO_4 (mg)	-----	-----	----	55
$MgSO_4 \cdot 7H_2O$ (mg)	11	22	----	220
KCl (mg)	2705	55	22	11
NaCl (mg)	27.5	55	55	----
$CaCl_2 \cdot 2H_2O$ (mg)	1.4	2.75	55	11
Yeast exteract(mg)	-----	-----	2.75	0.66
$FeSO_4 \cdot 7H_2O$ (μg)	27.5	27.5	-----	1.1
$ZnSO_4 \cdot 7H_2O$ (μg)	82.5	82.5	1.1	82.5
$MnSO_4 \cdot 7H_2O$ (μg)	82.5	82.5	82.5	82.5
H_3BO_3 (μg)	16.5	16.5	82.5	16.5
$CaCl_2 \cdot 6H_2O$ (μg)	8.3	8.3	16.5	8.3
$CuSO_4 \cdot 5H_2O$ (μg)	8.3	8.3	8.3	8.3
$NaMoO_4 \cdot 2H_2O$ (μg)	5.5	5.5	8.3	5.5
H_3PO_4 (γ = 1.71 g ml^{-1})(μl)	------	8.25	5.5	------

achieved when the values for dry mass, surface and interfacial tensions, and glucose and biosurfactant concentrations, remained constant during at least six volume changes.

Analytical methods

Biomass was determined in triplicate by centrifugation of 10 ml samples of culture liquid at 5.5 g during 25 min at room temperature. The cell pellet was washed once with distilled water, dried at 105 °C for at least 24 h, and then weighed. The supernatant was used for the estimation of glucose, surface and interfacial tensions, and biosurfactant concentration. Glucose was assayed in a YSI model 23A glucose analyzer (Yellow Springs, Ohio). The surface tension was measured with the same auto tensiometer. The surface-active rhamnolipids were isolated by liquid chromatography by the method of *Itoh* et al.[13] Rhamnose was identified by one-dimensional thin-layer chromatography on silica gel plates after acidic hydrolysis of the rhamnolipids. Three solvent systems were used:

(i) ethyl acetate: n-butanol : methanol : water (Ψ_m = 16:3:3:2) and ethyl acetate : acetic acid:methanol:water (Ψ_m = 6:1.5:1.5:1);

(ii) n-butanol: ethyl acetate:propanol:water (Ψ_m = 35:10:6:3) and acetone:water (Ψ_m = 9:1);

(iii) methanol:acetic acid:chloroform (Ψ_m = 15:5:80). Identification was performed by running rhamnose standards simultaneously.

The general procedure for downstream processing of the produced biosurfactant was developed for laboratory-scale investigations but was principally transferable to pilot plant scale. A scheme of the whole downstream process is given in Fig. 2. The outlet of the continuous production in the bioreactor was collected in a cooled reservoir tank (300 l). Periodically the cells were separated by centrifugation and the cell-free culture liquid was stored in a second reservoir.

Biosurfactant mass concentration was estimated by two methods, an indirect measurement and the determination of rhamnose mass concentration. The indirect estimation was based on the fact that the surface activity is dependent on the mass concentration of the active compound. When the concentration is below a certain value (critical micelle concentration [cmc] the surface activity is lost, which is expressed by increasing surface and interfacial tensions. This property was used for the estimation of the concentration of the active compounds. The culture broth was diluted until the interfacial tension increased and the dilution factor (F_{cmc}) was determined. Increasing F_{cmc} values indicate the increasing mass concentration of active compounds (Fig. 1). The other method was based

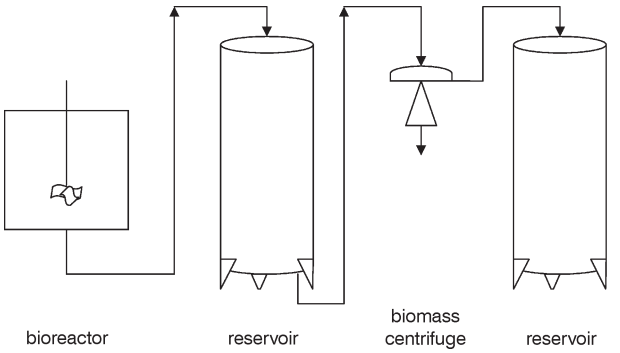


Fig. 1 – *Flow sheet for the production of rhamnolipid biosurfactants from P. aeruginosa.*

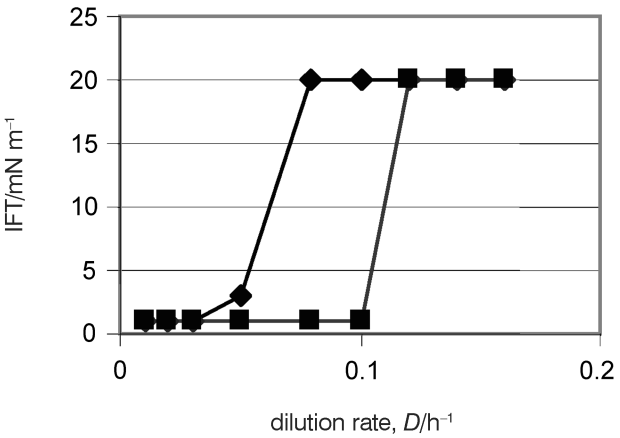


Fig. 2 – *Indirect measurement of biosurfactant concentration by determination of the F_{cmc}. Samples of the culture liquid were diluted until the interfacial tension (IFT) increased. Sample 1 (□) has an F_{cmc} of ca. 50, and sample 2 (◇) has one of 100. It follows that the concentration of the active compound is twice as high in sample 2 as in sample 1.*

on the determination of rhamnose by the following procedure. Cell-free culture broths were diluted to a rhamnose mass concentration of up to 25 μg ml^{-1} in 0.1 mol l^{-1} phosphate buffer (pH 7), and then a 1ml sample was extracted twice with 3 ml of diethyl ether. After evaporation of the organic phase, 0.5 ml of distilled water was added, and the rhamnose was quantified by the orcinol method.[2] The absorbance was measured at 420 nm, with rhamnose as the standard. Total organic carbon (TOC) content of the supernatant was assayed with a TOC analyzer (Rapid-C; Heraeus, Hanau, Federal Republic of Germany).

Results

Initial biosurfactant formation by *P. aeruginosa*. The isolated strain produced surface-active compounds when cultivated in batch cultures on medium 1M.[9] The minimum values for surface and interfacial tensions were 29 and 0.25 mN m^{-1}, respec-

tively, and a maximum concentration expressed as an F_{cmc} of 8 was obtained. The active compounds were analyzed by thin-layer chromatography as described by Itoh et al.[13] With the same solvent system, the surface-active components exhibited the Rf values established by these authors, i.e., 0.4 and 0.8. Further identification of the sugar moiety as rhamnose indicated that our *P. aeruginosa* strain most probably produced the same rhamnolipids as Itoh et al.[13] reported for their strain. The surface and interfacial tensions of a mixture of enriched rhamnolipids 1 and 2 (50 to 80 % pure) were 29 and 0.25 mN m^{-1}, espectively. Therefore, these values can be considered typical for the rhamnolipid biosurfactants. When the strain was cultivated in continuous culture, the minimum surface and interfacial tensions were 35 and 5.5 mN m^{-1}, respectively.[9] It followed that the properties of the spent medium were significantly less favorable with respect to surface activity in continuous culture. Since the composition of the medium influences the amount of the biosurfactant produced,[3,20] a medium optimization was performed in continuous culture.

Influence of nitrogen source and yeast extract on biosurfactant production.

Medium 2M with a glucose mass concentration of 18.2 g l^{-1} served as the basic medium for the optimization experiments. The influences of the nitrogen source (ammonium or nitrate) and the yeast extract on biosurfactant production were investigated by medium shifts in a continuous culture with a dilution rate of 0.1h^{-1}. With both nitrogen sources, biosurfactant production was poor when yeast extract was present in the medium (Table 2). Still, slightly better production was obtained with nitrate, as indicated by the lower surface and interfacial tension values of the culture broth, as compared for the cultivations in which ammonium served as nitrogen source.

A more pronounced influence was exerted by yeast extract. In its presence, biomass concentration was high and the substrate was completely used by the cells. When yeast extract was omitted, biomass concentration decreased and a moderate accumulation of glucose occurred, indicating a medium limitation other than carbon. It seems, that such conditions favored biosurfactant formation by the cells, since surface and interfacial tensions decreased to 29 and 0.25 mN m^{-1}, respectively, i.e., the typical minimum values observed in the initial batch culture. In addition, better production was noted in the medium containing nitrate as the nitrogen source. To estimate the dilution rate range for the intended optimization of the nutritional requirements for biosurfactant production, the experiments described above were also done at a dilution rate of 0.28 h^{-1}. With this dilution rate, the surface tension of the culture broth never dropped below 35 mN m^{-1}, indicating that fewer surface-active compounds were released by the cells at the higher dilution rate. As a consequence of these results, subsequent experiments were carried out at a dilution rate of 0.10 h^{-1} with nitrate as the sole nitrogen source.

A continuous with a dilution rate of 0.1 h^{-1} and medium 3M with a glucose mass concentration of 16.2 g l^{-1} were used. Abbreviations: S, residual substrate mass concentration; ST, surface tension: IFT, interfacial tension; F, factor yields concentration of the active compounds below the F_{cmc}, NA, not applicable, because ST and IFT values were already considerably above the characteristic low values of 29 and 0.25 mN m^{-1}, respectively, and, therefore, the concentration of the active compounds was already below the F_{cmc}.

Effect of carbon/nitrogen mass ratio

Aiming at increasing the production of rhamnolipids by *Pseudomonas aeruginosa*, a study with increasing glycerol volume fraction (φ_m = 1; 2; 3; 4; 5 and 6 %) was conducted and standardized inoculum of 0.1 g l^{-1} was employed. Figure 3 shows the yield factors relating substrate consumption to production ($Y_{P/S}$) and production to biomass ($Y_{P/X}$). The best results ($Y_{P/S}$ = 0.13 g g^{-1}; $Y_{P/X}$ = 0.70 g g^{-1}) were obtained when glycerol was used in a volume fraction of φ_m = 5 %, corresponding to a $\zeta_{C/N}$ mass ratio of 55 : 1. Additionally, it is possible to observe that the yield $Y_{P/S}$, decreased after this optimum glycerol volume fraction, reaching its lowest value

Table 2 – *Influence of nitrogen source and yeast extract on P. aeruginosa growth and biosurfactant production*

Nitrogen source $\gamma_{n/}$ g l^{-1}	Yeast extract γ_n / g l^{-1}	Biomass γ_n / g l^{-1}	S γ_s /g l^{-1}	ST σ_m / mN m^{-1}	IFT σ_m / mN m^{-1}	F_{cmc}
$(NH_4)_2SO_4$(4)	1.5	4.77	0.1	32	3.5	NA
	0	3.11	1.48	30	0.5	5
$NaNO_3$ (3)	1.5	5	0.1	30	3	NA
	0	3.35	0.48	29	0.25	10

($Y_{P/S}$ = 0.075 g g^{-1}) for the highest glycerol volume fraction (φ_m = 6 %), thereby indicating a possible inhibitory effect on the bacterium metabolism due to a likely nutrient transport deficiency.[6,7,8]

Influence of Fe mass concentration on growth and biosurfactant production.

Of the trace elements, Fe had a major influence on *P. aeroginosa* biosurfactant production. At high Fe concentrations, formation of surface-active components did not occur. To establish the correlation between biosurfactant production and Fe concentration, media with different C-to-Fe ratios were used in the continuous culture, and the influence on steady-state biosurfactant concentration was determined. In medium 2 mol l^{-1} with 18.2 g l^{-1} of glucose and the concentrations of all other components being constant, the Fe mass concentration was varied from 0.5 to 10 mg l^{-1} of $FeSO_4 \cdot 7H_2O$, yielding C to Fe ratios between ca. 3600 and 72400 (Fig. 2). The highest biosurfactant concentration (above 500 mg l^{-1}of rhamnose, corresponding to an F_{cmc} of ca. 70) was reached at a C-to-Fe ratio of 72.400 ($FeSO_4 \cdot 7H_20$ concentration of 0.5 mg l^{-1}). With decreasing C-to-Fe ratios, the biosurfactant concentration also decreased. At the lowest C-to-Fe ratio of 3.6, about 150 mg l^{-1} of rhamnose l^{-1} was present (corresponding to an F_{cmc} of 8). Biomass concentration decreased slightly above a C-to-Fe ratio of $\zeta_{c/Fe}$18.00, whereas no significant accumulation of glucose took place.

One of the released products was the rhamnolipid with a maximum formation around a C-to-N ratio of $\zeta_{C/N}$ = 18. Since glucose did not accumulate, it follows that the cellular metabolism was directed towards product formation by N_2 limitation.

Influence of phosphorous on growth and biosurfactant production

Phosphorus represents another important element in bacterial metabolism. To evaluate the influence of the phosphate concentration, media with 0.71 to 2.12 g l^{-1} of phosphate were prepared, covering the C-to-P ratio range from $\zeta_{C/P}$ = 10 to 32.

Biosurfactant formation of the cells remained at its maximum up to a C-to-P ratio of $\zeta_{C/P}$ = 16 (Fig. 3). When the media of higher C-to-P ratios were applied, a decrease in biosurfactant concentration occurred. Biomass concentration did not change significantly, indicating that there was no expressed P limitation on all the phosphate concentrations tested. A certain surplus of phosphate was apparently required for *P. aeruginosa* biosurfactant formation.

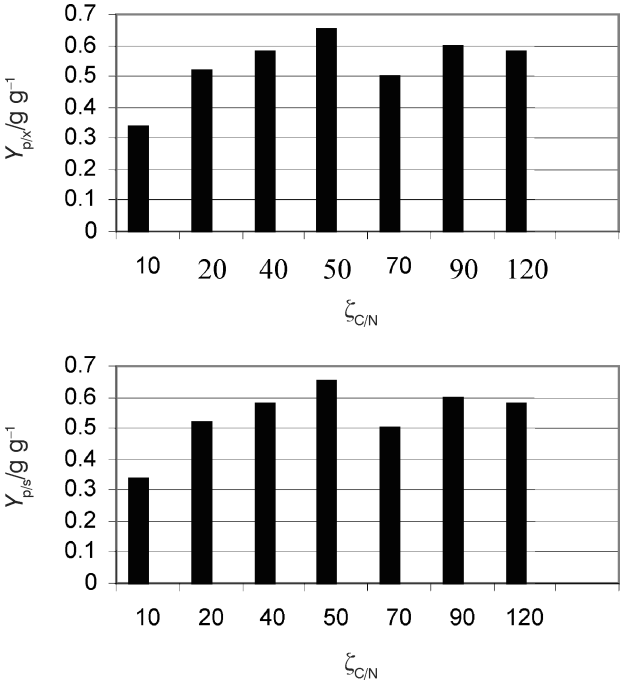


Fig. 3 – *Yields of rhamnolipids related to glycerol consumption ($Y_{p/s}$) for fermentations by Pseudomonas aeruginosa with different $\zeta_{C/N}$ ratios and Yields of rhamnolipids related to biomass ($Y_{p/x}$) for fermentations by Pseudomonas aeruginosa with different $\zeta_{C/N}$ ratios*

Growth and biosurfactant production with optimized medium as function of dilution rate

Based on the results of the optimization, medium 3M was formulated (Table 1). With this medium, a final analysis of biosurfactant production and growth as a function of dilution rate was performed.

Glucose mass concentration amounted to 18.2 g l^{-1}. The biosurfactant production was at its maximum up to a dilution rate of D_m = 0.15 h^{-1} (F_{cmc} of 68) (Fig. 5). Higher dilution rates led to a decrease in the biosurfactant and biomass concentrations, and simultaneously, glucose started to accumulate. The surface and interfacial tension values remained at their minimum up to a dilution rate of 0.23 and then increased with increasing dilution rates. With medium 3M, it was not possible to reach steady states at dilution rates greater than 0.32 h^{-1} Washout of the culture occurred above the mentioned dilution rate.

Effect of the nitrogen source

Table 3 shows that sodium nitrate ($Y_{P/X}$ = 0.7 g g^{-1}) is more effective than ammonium sulfate ($Y_{P/X}$ = 0.35 g g^{-1}) and urea ($Y_{P/X}$ = 0.5 g g^{-1}). As shown in this figure, the use of nitrate at a $\zeta_{C/N}$ ratio of 55 : 1 implies better productivity than use of ammonium at the same $\zeta_{C/N}$ ratio, using φ = 5 % of

Table 3 – *Effect of nitrogen sources on the production of rhamnolipids by Pseudomonas aeruginosa*

Nitrogen source	Production of rhamnolipid per mass unit of nitrogen source P_m / g $g^{-1}h^{-1}$
Urea	0.5
Nitrate	0.7
Ammonia	0.58

glycerol as carbon source. This result can be explained by the fact, that nitrate first undergoes dissimilatory nitrate reduction to ammonium and then assimilation by glutamine-glutamate metabolism. This means that assimilation of nitrate as nitrogen source is so slow that it would simulate a condition of limiting nitrogen[9,11,14]

Pseudomonas aeruginosa is able to use nitrogen sources such as ammonia or nitrate. However, in order to obtain high concentrations of rhamnolipids it is necessary to have restrained conditions of this macro-nutrient. Our studies showed, that nitrate is more effective in the production of rhamnolipids than ammonia and urea, which is in agreement with other studies reported in the literature.[5,7,9]

The *P. aeruginosa* strain, used in this study, produced rhamnolipids when grown in batch cultures with glucose as the carbon and energy source. In view of the possible use of biosurfactants, our goal was to develop a continuous production process. Such a process has several advantages as compared with the production of biosurfactants in batch cultivations:

(i) the long-term incubations of several days[3,14,19] are avoided, yielding a much improved productivity per unit of reactor volume;

(ii) there is a constant mass flow which can be adapted to the capacity of the downstream processing;

(iii) the exact control of the culture conditions which is essential for high biosurfactant formation by the cells, is accomplished more easily in a continuous culture.

For the design of the continuous process, it was not possible to take over the batch data directly. When the medium, which led to biosurfactant production in batch cultures was used in continuous culture, very poor formation of surface-active compounds resulted, and a medium optimization was necessary. Several medium components influenced the formation of rhamnolipids by the cells. Additionally, there was no unique pattern of how a particular component affected the performance of the cells. An optimal C-to-N mass ratio for nitrate concentration (Fig. 3), a minimal iron concentration (Fig. 2), and a surplus of phosphorous (Fig. 4) yielded high biosurfactant production. From the data of the biomass concentration and that of total organic carbon concentration in the spent medium (Fig. 3), it followed that a certain metabolic state needed to be achieved for a general product formation of the cells. By applying limitations other than carbon (iron and nitrogen here), it was possible to direct cellular metabolism to product formation. This seems to be a general concept, since medium limitations were also leading to polysaccharide production.[17,23] Because of these facts, the use of complex medium additives, such as yeast extract, has to be avoided. This became obvious in the case of biosurfactant production (Table 2). As far as the growth rate of the cells is concerned, production of the surface-active compounds was bound to low dilution rates. When it was raised above 0.15 h^{-1}, biosurfactant production of the cells dropped (Fig. 5). Again, this observation is in accordance with

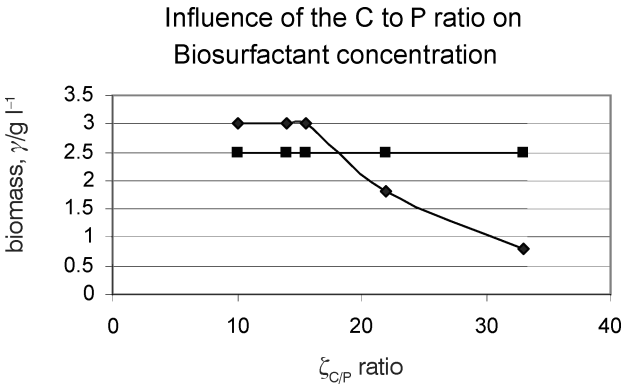


Fig. 4 – *Influence of the C to P ratio on biosurfactant concentration in P. aeruginosa cell-free culture liquid in a continuous culture with a dilution rate of D = 0.01 h^{-1}, Medium 2M with 18.2 g l^{-1} glucose and 2.5 g l^{-1} of $NaNo_3$ and 0.5 mg l^{-1} of $FeSO_4 \cdot 7H_2O$ was used .symbols ■, biomass ◆ rhamnose*

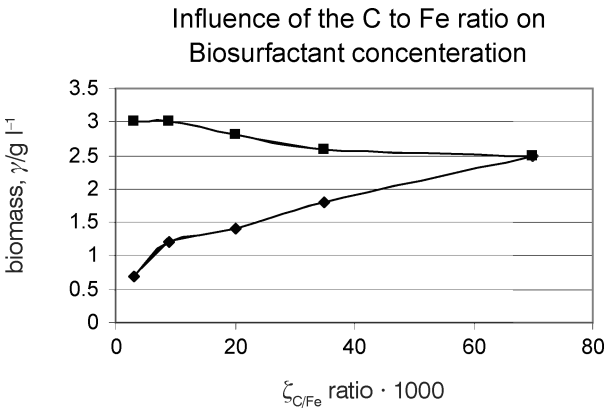
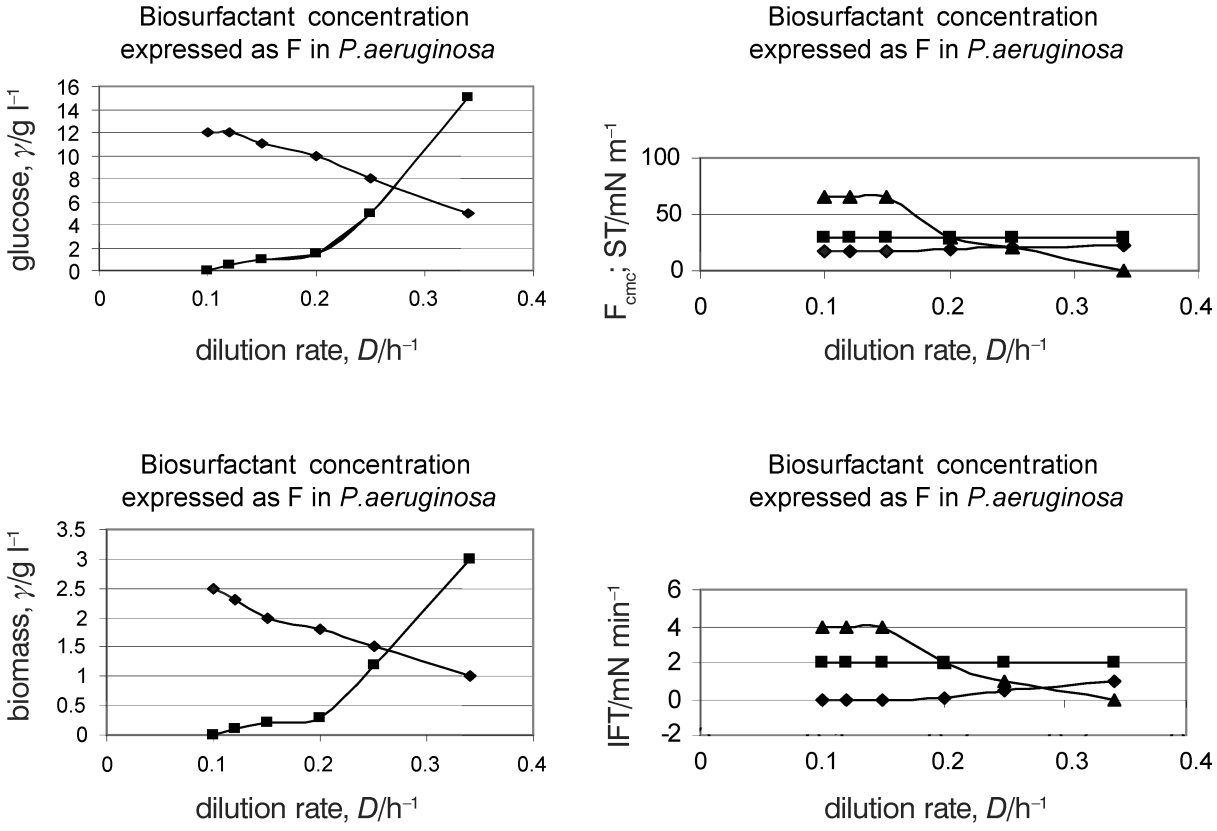


Fig. 5 – *Influence of the C to Fe ratio on biosurfactant concentration in P. aeruginosa cell-free culture liquid in a continuous culture with a dilution rate of D = 0.01 h^{-1}, Medium 2M with 18.2 g l^{-1} of glucose and 2.5 g l^{-1} of $NaNO_3$ was used .symbols: ■ biomass ◆ rhamnose*



F i g . 6 – *Biosurfactant concentration expressed as Fcmc in P. aeruginosa cell-free culture liquid as a function of the dilution rate. Medium 3 M with 18.2 g l^{-1} of glucose was used symbols: ■, biomass ◆ rhamnose ▲ Fcmc*

other reports on the production of extracellular microbial metabolites, which are also related to low dilution rates.[17,22,23] Our data indicates that by proper medium design the production of surface-active compounds in continuous culture is possible. The optimization performed here gave rhamnolipid concentrations of up to 1.5 g l^{-1} with a starting glucose mass concentration of 18.2 g l^{-1}. As compared with the concentration in the initial batch cultures, an almost 10-fold higher biosurfactant concentration resulted in the continuous process described in this study. This yield can certainly be increased by further process development or strain improvement.

List of symbols and abbreviations

cmc – critical micelle concentration
D_m – dilute ratio, h^{-1}
F_m – yield of the active compounds
IFT – interfacial tension, mN m^{-1}
n – stirring speed, min^{-1}
t_m – time, min
T_m – temperature, °C
V_m – volume, l
γ_m – mass concenteration, g l^{-1}
γ_{sm} – residual substrat concentration, g l^{-1}
$\xi_{m/m2}$ – mass ratio,
φ_m – volume fraction
$\psi_{v/v}$ – volume ratio
Y_m – yield, g g^{-1}
σ_m – surface tension, mN m^{-1}
m_m – mass, mg, g, μg

References

1. *Arima, K., Kakinuma, A., Tamura, G.,* Biochem. Biophys. Res. Commun. **31** (1968) 488.
2. *Chandrasekaran, E. V., Bemiller, J. N.,* Methods Carbohydrate. Chem. **8** (1980) 89.
3. *Cooper, D. G., MacDonald, C. R., Duff, S. J. B., Kosaric, N.,* Appl. Environ. Microbiol. **42** (1981) 408.
4. *Cooper, D. G., Paddock, D. A.,* Appl. Environ. Microbiol. **47** (1984) 173.
5. *Cooper, D. G., Zajic, J. E.,* Adv. Appl. Microbiol. **26** (1980) 229.
6. Department of Energy. International conference on microbial processes useful in enhanced oil recovery. CONF-790871. U.S. Department of Energy, Washington, D.C., October 1979.
7. *Edwards, J. R., Hayashi, J. A.,* Arch. Biochem. Biophys. **111** (1965) 415.
8. *Finnerty, W. R., Singer, M. E.,* Biotechnology **1** (1983) 47.
9. *Guerra-Santos, L., Kappeli, O., Fiechter, A.,* Growth and biosurfactant production of a bacterium in continuous culture In *E. C. Donaldson* and *J. B. Clark* (ed.). International

conference on microbial enhancement of oil recovery. Proceedings. CONF-820540. U.S. Department of Energy. Washington, D.C. 1983, p. 12–14.

10. *Hisatsuka, K., Nakahara, T., Minoda, T., Yamada, K.,* Agric. Biol. Chem. **41** (1977) 445.
11. *Hisatsuka, K., Nakahara, T., Sano, N., Yamada, K.,* Agric. Biol. Chem. **35** (1971) 686.
12. *Holdom, R. S., Turner, A. G.,* J. Appl. Bacteriol. **34** (1969) 448.
13. *Itoh, S., Honda, H., Tomita, F., Suzuki, T.,* J. Antibiot. **24** (1971) 855.
14. *Itoh, S., Suzuki, T.,* Agric. Biol. Chem. **36** (1972) 2233.
15. *Jarvis, F. G., Johnson, M. J.,* J. Am. Chem. Soc. **71** (1949) 4124.
16. *Kappeli, O., Finnerty, W. R.,* Biotechnol. Bio eng. **22** (1980) 495.
17. *Mian, F. A., Jarman, T. R., Righelato, R. C.,* J. Bacteriol. **134** (1978) 418.
18. *Matsufuju, M., Nagata, K., Yoshimoto, A., Biotechnology letters*, **19** (12) (1997) 1213.
19. *Maier, R. M, Soberon Chavez, G.,* Appl. Microbiol. Biotechnol. **54** (2000) 625.
20. National Science Foundation. The role of microorganisems in the recovery of oil. In Proceedings Engineering Foundation Conferences. NSF/RA 770201. National Science Foundation, Washington, D.C. 1976.
21. *Rapp, P., Bock, H., Wray, V., Wagner, F.,* J. Gen. Microbiol. **115** (1979) 491.
22. *Ristau, R., Wagner, F.,* Biotechnol. Lett. **5** (1983) 95.
23. *Suzuki, T., Tanaka, H., Itoh, S.,* Agric. Biol. Chem. **38** (1974) 557.
24. *Toda, K., Takeuchi, T., Sano, H.,* J. Chem. Tech. Biotechnol. **29** (1979) 747.
25. *Williams, A. G., Wimpenny, J. W. T.,* J. Gen. Microbiol. **104** (1978) 47.